OBSERVATIONS ON ANTIBIOTIC RESIDUES IN MILK

J. E. KAJUMIS

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JULIUS KITHINJI KAJUME

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Royal (Dick) School of Veterinary Studies
Department of Animal Health
Veterinary Field Station
Easter Bush
Roslin
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ABSTRACT

The excretion of oxytetracycline and penicillin in bovine milk following their intramuscular administration was demonstrated by bromocresol purple test ("Intertest"). Dosages of 1200 mg of oxytetracycline hydrochloride and 5 mega units of penicillin (crystapen) per cow both resulted in detectable residues in milk for 24-32 hours following administration. The minimum concentration of oxytetracycline in the serum which resulted in detectable residues in milk was 2.5 mg/ml while that of penicillin was 0.16iu/ml. Antibiotic concentrations in the serum were determined by the tube dilution method.

The effect of heat (boiling at 100°C for 10 minutes) on known concentrations of oxytetracycline and penicillin added to milk was determined using a modified disc assay method with the Oxford (Heatley) Strain of Staphylococcus aureus (NCTC 6571) as the test organism. Oxytetracycline was partially inactivated with a mean antibiotic activity loss of 37.8% whereas penicillin was not inactivated to any noticeable extent.

INTRODUCTION

The value of antibiotic usage in both veterinary and human medicine cannot be underrated. Loss of life would be enormous without the use of antibiotics. Diseases which would otherwise have devastating effects on both human and animal populations have been combatted through the use of antibiotics and other antibacterial agents. In countries where the availability of antibiotics and other drugs are either lacking or inadequate, diseases cause excessive suffering to people and form a formidable barrier to development.

Further, the demand for higher productivity in animal production to satisfy the needs of the ever increasing world population calls for a wider scope of antibiotic utilisation. An example of this is the use of some antibiotics as growth promotants especially in poultry and pig farming. In addition, intensive livestock farming, which is common in many agricultural communities, can create stress to animals and enhance spread of various diseases within the herd subsequently resulting in an increased usage of antibiotics and/or other chemotherapeutic agents. To a lesser extent antibiotics, such as oxytetracycline and chlortetracycline, have been used as food preservatives.

From the above information it is evident that the antibiotics are almost indispensable and will perhaps remain so for years to come.

However, side effects resulting from the widespread use of antibiotics in animals and man have been described by many research workers. Of great concern has been the development of microbial populations resistant to some of the antibiotics used, making it almost impossible to adequately treat a person or animal in

infections where such antibiotics are indicated. Allergic reactions resulting from frequent contact with small amounts of these compounds have also been reported. Specifically, the ingestion of minute quantities of antibiotics, mainly penicillin, in the form of residues in food has been found to cause allergic manifestations in sensitised individuals. It is therefore necessary to protect the consumer from frequent contact especially where undue and undesirable forms of contact are concerned. Thus, a situation has arisen demanding the investigation of the occurrence of antibiotic residues in foods of animal origin, their possible health hazards and the formulation of appropriate remedial measures. Both the experimental work and the review in this dissertation were formulated to meet this demand.

Milk is one of the most important food commodities requiring attention as far as antibiotic residual problems are concerned. Antibiotics have been shown to be excreted in the milk following their administration to dairy cows. However, the information available on the presence of residues in milk following administration by injection is comparatively scanty and inconclusive. In this dissertation one of the objectives was therefore to obtain more information about the excretion of antibiotics in milk following parenteral administration. In the review attention was given to various parenteral routes including a brief mention of intramammary and intrauterine infusions but in the experiment, because of various practical limitions, the scope was narrowed to intramuscular administration only. Nevertheless intramuscular administration of antibiotics is quite common and is of practical importance in treatment of animal diseases.

In the experiments described oxytetracycline, a broad spectrum antibiotic, and penicillin were used. According to Fortunio (1977) the use of broad spectrum, as compared with narrow spectrum, antibiotics has increased considerably. The fact that they are indicated for a greater variety of infections than any other antibiotic encourages their choice particularly in many cases where a bacteriological diagnosis is unavailable. Penicillin was chosen because, whilst according to many research workers it is the most frequently used drug in the treatment of mastitis in dairy cows and therefore likely to be found in milk, its allergenicity in low concentrations constitutes a public health hazard meriting continual assessment.

Several methods of overcoming the problem of antibiotic residues in milk and other foods have been suggested and attempted. Heat treatment has been tried by a few workers but the information available so far is not completely conclusive. In view of the fact that boiling of milk before consumption is a common practice in many parts of the world today it was considered appropriate and necessary to investigate the effect of such treatment on oxytetracycline and penicillin residues in milk.

The purpose of the study

The purpose of this study was to review the literature on antibiotic residues in milk, mainly oxytetracycline and penicillin, with particular emphasis on their excretion in milk following parenteral administration and their possible health hazards to humans. Experiments were designed and conducted to determine:-

(1) whether or not (a) oxytetracycline and (b) penicillin are excreted in bovine milk following single intramuscular injections and the duration of detectability of residues by the modified disc assay and bromocresol purple (intertest) methods;

(2) the correlation between concentrations in the serum and the duration of detectability of residues in milk; and

(3) the effect of heat (boiling) on such residues in milk.

THE REVIEW OF THE LITERATURE

1. Antibiotic Residues in Milk Following Parenteral Administration to Dairy Cows

The excretion of antibiotics in milk following their administration by parenteral routes has been reported in the literature but whereas the excretion is greatly influenced by the route of administration other factors play a part as well.

The antibiotic mainly researched and reported on is penicillin.

1.1 Excretion following intramuscular administration: Edwards and Haskins (1953) showed that penicillin, aureomycin and streptomycin at the dosage of 11 mg/kg body wt were excreted in milk but whereas aureomycin and streptomycin were detected in the milk and blood over the same period of time, penicillin was detected for a longer period in milk than in blood. Sadek (1954), using penicillin (pronapen) at the rate of 5000 units/lb body wt in a single dose, demonstrated that residues were readily detectable in milk for a period of 24 hours. However, Williams and Laverne (1960) showed that, when penicillin in aqueous suspension was used at the rate of 2000 and 5000 units/lb body wt, residues persisted in milk for 48 hours and 3 days respectively. Sadek (1954) also observed that following either intramuscular or subcutaneous administration penicillin appeared in the milk within a shorter time and in higher concentrations in cows with mastitis than in normal cows.

Krawczyk and Olson (1961), while reporting on the presence of penicillin residues in milk following intramuscular injections of 3000000 units/cow, observed that, in low milk producers, the drug was excreted in milk for a longer period of time than in high producers.

The maximum times residues appeared in milk were 48 hours and 24 hours for low and high milk producers respectively. Doubling the dosage increased these periods to 108 hours and 36 hours respectively. Mol (1975), using benzathine penicillin, found that 3000000 iu/cow resulted in a clearance time of 6.5 days with a peak concentration of 0.2 iu/ml of milk while doubling the dose resulted in a clearance time of 13 days with a peak concentration of 0.5 iu/ml of milk. In another experiment Mol (1975) found that after intramuscular injection of 3000000 iu sodium benzylpenicillin per animal the residues were detectable in the milk within 5 minutes and reached a peak concentration of 0.3 iu/ml. The clearance time was approximately 10 hours.

The base or the vehicle in which the antibiotic is suspended can influence its excretion in milk. Demonstrating this Canon, Hawkins and Wiggins (1962) used penicillin, at the rate of 3000 units/lb body wt, in an oil base resulting in detectable residues in milk for a period of 96 hours while the same dosage in an aqueous base resulted in detectable residues for 72 hours.

Edwards (1966) found that procaine penicillin and penethamate (undissociated form of penicillin), when administered at the rate of 1-5 mega units per cow, were excreted in milk but the levels following penethamate injection were approximately 5 to 10 times greater than those resulting from procaine penicillin.

Dihydrostreptomycin, when injected at the rate of 0.5 gm/50 or 100 lb body wt was detected in milk in concentrations of 0.05 to 0.125 mg/ml 12 hours post injection with a clearance time of 36 hours (Brobel and Burch, 1960a).

Williams and Laverne (1960) demonstrated that following a single dose of 2 mg of tetracycline hydrochloride per lb body wt residues were detected in milk for 30 hours. Almost similar findings were obtained by Mol (1975) who showed that, after a single dose of 1.25 gm of an aqueous solution of oxytetracycline per animal, residues appeared in milk within 6 hours with a clearance time of 2 days.

Chloramphenicol diffues easily from the blood-stream into the milk (Mol 1975). He injected 5 gm of an aqueous solution of chloramphenicol resulting in detectable residues in milk in less than 5 minutes with a peak concentration of 4 mg/ml in 30 minutes and a clearance time of approximately 3 hours.

Absence of antibiotic residues in milk following intramuscular administration has also been reported by some workers. Slavin (1946) was unable to detect penicillin residues in milk of two cows with mastitis following injections of 114000 and 128000 units respectively. A similar observation was made by Watts and McLeod (1946) using a dosage of 100000 units and a test of sensitivity 0.02 unit/ml of milk. Dosages of up to 6000000 units of procaine penicillin per cow, using tests of sensitivity 0.05 unit/ml of milk, did not result in detectable residues in milk (Albright, Ormiston, Brodie and Witter 1961; Albright, Ormiston, Brodie and Witter 1962).

Barnes (1956) found that 1 gm of oxytetracycline per cow did not results in detectable residues in milk. Schipper and Petersen (1953) administered 3 gm of terramycin but were also unable to demonstrate the presence of residues in milk.

1.2 Excretion following intravenous administration: Edwards and Haskins (1953) showed that penicillin, aureomycin and streptomycin

at the dosage of 11 mg/kg body wt. were excreted in milk following intravenous as well as intramuscular administration while Canon et al (1962) detected penicillin residues in milk following intravenous administration of 2000 units/lb body wt for a period of up to 44 hours post injection.

Schipper and Petersen (1953) detected terramycin residues in milk 1 to 2 hours after administration of 5 mg/lb body wt. Smaller dosages of tetracyclines also result in detectable residues in milk. Blobel and Burch (1960b), using 2-4 mg of oxytetracycline per lb body wt. detected residues in milk but all samples were free from detectable residues 24 hours after administration. The sensitivity of the test used was 0.05 µg/ml of milk. On the other hand Williams and Laverne (1960) injected tetracycline hydrochloride at the rate of 2 mg/lb body wt resulting in demonstrable residues in milk up to 30 hours post injection, while Blobel and Burch (1960b) administered chlortetracycline at the same dosage and detected residues in milk for a period of 48 hours following administration. Hokanson, Watrous, Burch and Eberhart (1963) found that, after intravenous treatment of 9 cows with tetracycline, bacterial inhibition occurred in bucket milk for approximately 36 hours after treatment.

Few workers have reported on the absence of antibiotic residues in milk following intravenous administration. Seeley, Anderson, Plastridge and Patricia (1945) used the disc assay method with Staphylococcus aureus H as the test organism and were not able to detect penicillin in the milk following intravenous injections of 80000 or 500000 units. Barnes (1956) injected each of two cows with 1 gm of oxytetracycline but did not detect residues in the milk.

1.3 Excretion following Subcutaneous Administration: Welsh, Langer, Burhart and Schroeder (1948) were among the first workers to detect penicillin residues in milk following parenteral (other than intramammary) administration. Their observations were made on two cows which received individual doses of 16250000 and 17250000 units of penicillin subcutaneously resulting in milk levels of 0.06 and 0.1 unit/ml respectively with traces still present 30 hours post administration. Sadek (1954), using a dosage of 5000 units of penicillin (pronapen) per lb body wt, detected residues in the milk for a period of approximately 24 hours after injection. These findings have been confirmed recently by Mol (1975) who, after subcutaneous administration of 3000000 iu procaine benzylpenicillin in aqueous solution per animal, found residues in milk within 1 hour of administration, a peak concentration of 0.2 iu/ml within 6 hours and a clearance time of 2 days.

1.4 Excretion following intramammary infusion: Intramammary infusion of antibiotic preparations is the most popular form of therapy in the treatment of mastitis in dairy cows. It has also been shown to be the main source of antibiotic contamination of milk.

Many research workers have shown that most of the antibiotic preparations used for the treatment of mastitis in dairy cows are excreted in milk following intramammary infusions. However, the concentration and the duration of detectability of residues in milk is influenced by several factors. Doan (1950); Claybauch and Nelson (1951); Marth and Ellickson (1959) indicated that the amount of antibiotic excreted in milk following intramammary infusion depends on the number of quarters treated, dosage used, vehicle carrying the

antibiotic, time elapsing after treatment and the level of milk production. These findings have been confirmed by other workers. Schipper and Petersen (1953) demonstrated that terramycin in an ointment vehicle resulted in a longer duration of excretion than in an aqueous vehicle. Albright, et al (1961) made a similar observation with penicillin while Caruolo, Clark, Jezeski, Menz, Messer, Miller, Smith and Wessen (1977) indicated that antibiotics in oil bases resulted in lower concentrations in milk but longer periods of excretion than in aqueous bases.

Investigations conducted by Mol (1975), using penicillin in two different dosages and in different carriers, revealed that the dosage has very little influence on the duration of residues in milk. It was shown that the major determinant factor was the carrier composition.

In his investigation Barnes (1956) found that all quarters had detectable levels of oxytetracycline for 24 hours or longer following a single 400 mg infusion. The sensitivity of the test was 1.25 µg/ml of milk. He also found much variation in the concentrations obtained in different cows and in different quarters of the same cow. The milk obtained from the quarter with the lowest daily milk production showed the highest level and the longest duration of detectability of oxytetracycline residues. Thus a correlation between the volume of milk production, and therefore the stage of lactation, the concentration and the duration of detectability of antibiotic residues in milk was established. This inverse correlation between the milk yield and the concentrations of the antibiotics in the milk following their administration to dairy cows has been reported by some other workers (Jackson and Bryan 1950; Blobel and Burch 1960b; Blobel 1960; Raemy 1977).

Transfer of antibiotics from treated to untreated quarters has been demonstrated. The concentration of the antibiotic in milk from the untreated quarters was found to be proportional to the total dose of infusion (Blobel and Burch 1960b; Blobel 1960; Albright et al 1961; Albright, Tuckey and Woods 1961; Caruolo et al 1977).

Hunter (1949a) showed that successive treatments did not result in a substantial build-up of penicillin concentration in milk.

Concentrations of the antibiotics in milk following intramammary infusion are generally higher than those resulting from intramuscular, intravenous or subcutaneous injections. Hargrove, Walter, Malkames and Maskell (1950) were able to recover 26 to 49% and 39 to 58% of the infused penicillin and streptomycin in milk respectively. Raemy (1977) infused vetramycin at the rate of 1200000 units/quarter, and on the average, 21.8% was found in milk. Values as high as 150 units of penicillin per ml can be found in milk 8 hours after administration of therapeutic doses (Caruolo et al 1977). Penicillin was still detectable at 80 hours, although at very low concentrations.

Overby (1952), using acid production by starter cultures as the main criterion, was able to demonstrate inhibitory activity of aureomycin, streptomycin, penicillin, terramycin and chloromycetin in milk obtained from the cows following treatment.

1.5 Excretion following intrauterine infusion: Penicillin (aqueous form) residues have been found in milk following intrauterine infusion of 1000000 units/cow but in only 16.7% of the animals tested (Canon et al 1962). Righter, Mercer, Kline and Carter (1975) showed that penicillin, dihydrostreptomycin and oxytetracycline

were excreted in milk but in trace amounts following intrauterine infusions of 2000000 units, 2.5gm and 500mg respectively.

Antibiotic concentration in milk following intrauterine application depends on the type, form and dosage of the drug used. Miller, Berght, Rose, Brunson and Messer (1973) observed that, following intrauterine administration of chelated and non-chelated forms of oxytetracycline, the latter appeared in milk 4 hours after administration whereas the chelated form was not detectable. This observation was confirmed by Miller and Berght (1976) who, using oxytetracycline at the dosage of 4 mg/kg body wt. found that the non-chelated form appeared in milk in concentrations of 0.4-0.1 µg/ml and remained throughout a 24 hour period while the presence of the chelated form was not demonstrable. Caruolo et al (1977) were also unable to demonstrate the presence of oxytetracycline (chelated form) residues in milk following administration. However, they detected benzylpenicillin in milk at the concentration of 10.7 iu/ml. Black, Mackay, Doig and Claxton (1979) have demonstrated the presence of procaine penicillin G, oxytetracycline, chloramphenicol-dapsone, reverin, acriflavin and hibitane residues in milk following intrauterine infusion. The range of concentration of the drug residues in milk was from 0.04 units/ml for penicillin to 200 µg/ml for hibitane.

2. Incidence of Antibiotic Residues in Milk Supplies - extent of the problem

Antibiotic contaminated milk from a single cow may lead to the bulked sample for the farm failing an antibiotic test depending on the degree of dilution and therefore the total number of milking cows. Surveys of milk supplies (bulked farm milk) have been carried

out in some countries in an attempt to obtain an accurate information on the incidence of antibiotic contamination of milk and in some cases it has been shown to be greater than could have been imagined. Examples from Great Britain and elsewhere are used to illustrate this point.

Great Britain: Great Britain is one of the countries where the supply and usage of antibiotics and other drugs are under relatively strict ethical and legal controls. Further, the producer's milk, which is handled by a milk marketing board, is tested for the presence of antibiotics and/or other inhibitory substances and, where found, the producer suffers heavy economic penalties. The test used has a 'fail' standard of 0.02 iu of penicillin or its equivalent per ml of milk.

In a survey carried out in England in 1951 on two groups of churn milk it was found that 1.4% of samples in one group and 2.8% in another contained penicillin (Storrs and Heitt-Brown 1954).

The Report of the Milk Hygiene Subcommittee of the Milk and Milk Products Technical Advisory Committee (Provan, Chalmers, Coffin, Cuthbert, Dyson, MacWalter, Morgan, Pinkerton, Ross, Neish and Scarlet 1963) revealed that in a survey conducted in 1961 11% of more than 41700 samples examined in England and Wales contained antibiotics, with a higher incidence in Winter than in Summer. There were also variations from one part of the country to another. In Scotland 9.9% of more than 2700 samples examined were also found to contain antibiotics. Penicillin was the antibiotic most commonly found in the survey.

The England and Wales Milk Marketing Board Testing Scheme was introduced in October 1965 and annual failure rates since then have been as shown below (Longstaff 1980)

Year	Test Failures as a percentage of tests
1966	1.2
1967	1.1
1968	1.0
1969	0.9*
1970	0.8
1971	1.0
1972	1.0
1973	1.0
1974	1.2
1975	1.1
1976	1.6**
1977	1.5
1978	1.2
1979	1.3

* A Report of the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine (Swann Report 1969) recommended more strict control measures on the supply and use of antibiotics in the veterinary field.

** Prior to July 1976 a test with a "fail" standard of 0.05 iu of penicillin or its equivalent per ml of milk was used.

United States: In a survey conducted in New York State in 1951, 1794 samples of fresh pasteurised milk were examined for lactic starter inhibitory substances. Approximately 7% were found to contain inhibitory substances, and where present, antibiotics ranged between 0.05-0.1 unit of penicillin or its equivalent per ml of milk (Kosikowsky, Henningson and Silverman 1952).

Grove (1959) reported on three market surveys conducted by the Food and Drug Administration in the period 1954-56. 94 samples were collected in the first survey, 474 in the second and 1706 in the third and the percentages of positive samples for penicillin and other antibiotics were 4.3, 11.8 and 6.9 respectively.

Ten surveys covering a 9 year period prior to 1960 showed that 5.2% of the total 7201 milk samples analysed were positive for the presence of antibiotics. However, only 0.54% of the total 770000 producer milk samples examined in 1960 were positive for antibiotics but this was after the control programmes had been implemented (Albright et al 1961).

In a 1972 national survey only 0.3% of the 220000 producer milk samples contained inhibitors (Caruolo et al 1977).

Spain (Southern Region): An analysis of 1346 milk samples showed that 32.6% of the samples contained non-specific inhibitory substances. Penicillin was detected in 1.2% of the samples; streptomycin 1.1%; bacitracin 9.8%; neomycin 1.0%; tetracyclines 1.0%; kanamycin 0.3% (Pozo Lora, Herrera Marteachie, Polo Villar, Lopez Gimenez, Jordal Villarejo and Iglesians Perez 1977).

3. Some Public Health Aspects of Antibiotic Residues in Milk

3.1 Hypersensitivity: It is strongly suggested and in some instances proven that antibiotics in minute amounts in foods can elicit allergic reactions. Malten (1968) indicated that the incidence of drug allergies does not depend on the doses administered but is a function of the duration and/or frequency of administration of the drug, its chemical structure and the site of the body with which it comes in contact.

The medical expert opinion on the subject is that the presence of any antibiotic in milk is undesirable on account of possible hazards including hypersensitivity reactions which can result from the consumption of milk containing very small quantities of penicillin

(Provan et al 1963). In the United States, the evidence gathered from 30 authorities in the field of antibiotics, therapy and paediatrics, indicated that penicillin is a highly active antigenic substance which even in small quantities can cause reactions in highly sensitised individuals (Welch 1957; WHO Technical Report 1968). It has been indicated that penicillin is the major offender in hypersensitivity reactions (Albright et al 1961; Malten 1968). Coleman and Siegel (1955) described a case of anaphylactic shock after a patient was injected with hormone using a cleaned and sterilised syringe which had previously been used to inject another patient with penicillin preparations. Passively sensitised sites of the patient responded to a penicillin dilution of 0.0005 unit.

It is stated (Siegal 1959) that as little as 40 iu benzyl-penicillin taken orally may elicit allergic reactions and that skin reactions do occur after consumption of milk containing traces of penicillin. Antibiotic residues in milk and milk products have on many occasions been implicated as the cause of allergic reactions in some people. Zimmerman (1957/58) treated 52 patients showing allergic reactions to penicillin. Two of these produced allergic manifestations after ingesting penicillin in milk and ice cream but showed favourable response to penicillinase therapy thus ruling out allergy to the dairy products themselves. Erskine (1958) described a patient who had a history of penicillin dermatitis following penicillin therapy and who developed allergic dermatitis after taking a milk diet which contained 0.06 unit of penicillin per ml. Zimmerman (1959) reported on 4 cases of chronic urticaria associated with the ingestion of dairy products containing penicillin. In another instance a patient who had a persistent oral and cutaneous blister-like lesions recovered without therapy when milk and other dairy products were

eliminated from the diet (Rasonove 1960).

Penicillin in concentrations of 0.01-1.5 unit/ml has been detected in bulked milk (Provan et al 1963) and such small quantities are large enough to induce recurrences in sensitised individuals (Malten 1968). In fact dermatitis caused by penicillin in bulked milk supply has been reported (Borrie and Barret 1961). In this case the already sensitised individual relapsed after ingesting milk with a penicillin content of 0.03 unit/ml but remained symptomless on penicillinase therapy.

A case in which an individual ingesting approximately 4000 units of penicillin in milk daily and who developed urticarial type of reaction when given 4 units of penicillin G intramuscularly was reported by Vickers, Bagrutuni and Alexander (1958).

Suggestions have been made that penicillin in milk may play a part in the increased number of humans who are sensitive to penicillin injections (Bryan 1951; WHO 1968). However, although the consumption of milk containing penicillin will elicit allergic reactions in hypersensitive individuals, there is no documented evidence that consumption of milk or milk products containing penicillin can alone induce the hypersensitive state (Mol 1975; Olson and Sanders 1975).

Antibiotics other than penicillins can also cause allergic reactions. Streptomycin is known to cause allergic manifestations but the small doses likely to be present in foods are not of major concern nor are they regarded as dangerous to humans (Van Keulen 1965; WHO 1968).

A small proportion of patients treated with chloramphenicol develop fatal aplasia of bone marrow and sensitisation by previous exposure may be responsible for this. Such an exposure may result from the consumption of small quantities in milk (WHO 1968). Expressing a similar opinion Garrod (1964); Van Keulen (1965) indicated that

chloramphenicol is a potential hazard and that its residues in food should be avoided.

Of the 8324 dermatological patients reported by Malten (1968) 24 were ascribed to penicillin, 21 to streptomycin and 2 to chloramphenicol.

Sensitisation to tetracyclines is rare and involves no risk of allergic effect (Garrod 1964; Van Keulen 1965; WHO 1968). Generally, broad spectrum antibiotics are poor sensitisers (Welch 1957). Oleondomycin, neomycin and novobiocin can cause hypersensitivity but to a very small extent (WHO 1968).

Findings and reports of several workers summarised by Mol 1975 showed that residues of penicillin/streptomycin combinations and tetracyclines are frequently found in fairly large amounts in foods of animal origin but that the antibiotics most connected with allergic reactions are penicillin, streptomycin, chloramphenicol and novobiocin.

Despite the above findings the population at risk is not large. When penicillin first became available for human use physicians found that approximately 3% of patients were allergic to penicillin, although by 1951, 10-12% of children who had not previously been given penicillin injections were sensitive to penicillin (Bryan 1951). Only up to 10% of the USA population is allergic to penicillin (Welch 1957). However, the incidence of antibiotic allergies may be higher than is reported in the literature (FAO /WHO 1969). This may be partly due to a lack of investigative procedures (Malten 1968; Swann 1969). This is a supplement to an earlier observation by Vickers (1964) who suggested that many unresolved cases of chronic urticaria may be associated with penicillin in milk and that this must always be considered in any case of this kind.

3.2 Development of resistant strains of micro-organisms as a result of exposure to the antibiotics: It is widely accepted that drug resistant variants are formed by a process of spontaneous mutation (mutation theory) and occurs in any sizeable population of micro-organisms even before contact with any drug. However, the resistance does not become apparent until the microbial population is exposed to antimicrobial agents. The concentration of the antibiotics and the time of exposure required to initiate this process should be high enough to inhibit the great majority of the sensitive organisms but not too high as to partially or completely inhibit the resistant mutants as well (WHO 1968; WHO/FAO 1969; Mol 1975).

Swann (1969) indicated that the development of resistance is encouraged by the use of subtherapeutic concentrations of antibiotics or by prolonged exposure. Demonstrating that continuous non-therapeutic use of drugs has been responsible for a substantial increase in resistant organisms of farm animals Siegel, Huber and Enloe (1974) observed 10% or more of antibiotic resistant organisms in farm cattle continually fed rations containing various drugs whereas resistant strains could not be detected in the majority of animals which had minimal exposure to antimicrobial drugs. This view had been expressed earlier by Watanabe (1971) who stressed that the use of chemotherapeutic agents as feed additives for animals poses a bigger problem than the therapeutic use because in the former the drugs are routinely fed to a greater number of animals more frequently whereas in the latter drugs are given only to a limited number of animals. Loken, Wagner and Henke (1971) and Smith (1967) have also shown that the use of antibiotics as food additives is partly responsible for the emergence of antibiotic resistant strains of bacteria in farm animals. It has further been indicated that the uncontrolled use of antibiotics

has had a major impact in the development of antibiotic resistant micro-organisms in farm animals (Kariuki 1977a; Smith, 1967). The low prevalence of resistant strains of Eschericia Coli in wild as compared to domestic animals is an indication of the role that animal husbandry, especially the use of antibiotics in animal feeds, may have in producing the high isolation rate of resistant strains from livestock (WHO 1978).

Therapeutic uses of drugs also result in the emergence of resistant strains (Smith 1958; Loken et al 1971).

Datta (1969) suggested that the use of antibacterial drugs in medicine has exerted a selective pressure in the dissemination of resistant determinants (R-factors) in the normal bowel flora. Similarly Watanabe (1971) suggested that the use of chemotherapeutic agents in man is more responsible for R-factors in human bacteria than the use of chemotherapeutic agents for animals. In support of this Richmond (1980) stated that the human use of antibiotics must have had an impact. In contrast Mouton, Glerum and Loenen (1976), in a seven-year survey (in man), showed that in some cases there was no correlation between the resistance rates of hospital isolates with the data on antibiotic usage.

The acquired drug resistance is transferable. Since its recognition in Japan in 1959/60 (Swann 1969) transferable drug resistance has been demonstrated in many instances (Smith 1966; Smith 1969; Loken et al 1971; Linton, Patrichia, Richmond, Gillespie, Rowland and Baker 1972; Howells and Joynson 1975; Kariuki 1977b).

The possibility that the resistant strains may pass from animals to man has been suggested (WHO 1968; Pohl and Thomas 1977). In fact the findings of Linton et al (1972) and Siegel, Huber and Drysdale (1975) did

indicate that the enteric floral of human beings in contact with farm animals contain greater frequencies of resistant organisms than do florae of people unexposed to farm animals.

Where man and animals share a common pathogen, the administration of antibiotics to animals encourages the prevalence of resistant pathogenic micro-organisms in man (Swann 1969). It was indicated that most cases of salmonella food poisoning are derived either directly or indirectly from animals through butcher's meat, poultry, milk and knacker's meat and this provides a means by which antibiotic resistance from animals can be transferred to man. Anderson (1968) reported an epidemic with a multi-resistant Salmonella typhimurium phage 29 which had been transferred from animals to man. Pohl (1977) estimated that 1-4% of human salmonella probably have a bovine origin and are multi-resistant. Multi-resistant strains of salmonella phages 204 and 193 which appeared in calves in 1977 (Great Britain) have already spread to the human food chain (Threlfall, Ward, Ashley, Rowe 1980).

As far as the influence of antibiotics on the development of resistant strains is concerned the main emphasis by many workers has been "prolonged and continuous exposure". The extent to which antibiotic residues in milk and/or milk products and other foods meet this criterion to influence the development of the resistant microbial population is a matter of speculation. However, there are some indications that small quantities of antibiotics can contribute to the problem.

Van Keulen (1965) showed that small quantities of antibiotics can influence the development of resistant strains. Amongst the intestinal flora, Coliform bacteria developed resistance to chlortetracycline and oxytetracycline when these antibiotics were

applied for a long period of time at the rate of 0.2-10 ppm.

Fluharty (1965) showed that even under natural conditions enough antibiotic residues can be inhaled to suppress the nasal flora and facilitate the establishment of antibiotic resistant staphylococci which can then be a reservoir of infection. Albright et al (1961) suggested that unintentional consumption of small amounts of antibiotics in foods may result in the development of resistant micro-organisms.

Salmonnella typhimurium in dairy calves receiving 80-100 ppm of tetracycline in their milk diet developed resistance (WHO 1968).

Mol (1975) indicated that good therapeutic and prophylactic regimes can hardly give rise to resistance problems but that residues in food and other antibiotic contaminations, such as feed additives, frequently reach the critical level which favours the selection of resistant strains.

Pohl (1977) studied the effect of tetracycline, chloramphenicol, neomycin and ampicillin upon the gut flora of pigs when added to feeds at the rate of 15-20 ppm. Tetracycline (T), chloramphenicol (C), ampicillin (A) and neomycin (N) led to the predominance of strains resistant to streptomycin (S) and T; C, T, and S; S, T, N and A; S, T, C, N and A respectively.

Thatcher and Simon (1955) found that 67% of streptococci and 15% of micrococci isolated from cheese were resistant to penicillin. The isolates were most often resistant to penicillin and dihydrostreptomycin, the two drugs commonly employed in the treatment of mastitis in cows. Marth and Ellickson (1959) suggested that dairy products made from milk containing antibiotic residues may contain unusual antibiotic resistant bacteria capable of causing illness in man.

It has been stated that micrococci and streptococci resistant to antibiotics are encountered in patients with no history of hospitalisation or antibiotic therapy and the cause may be found in the consumption of foods that contain such antibiotic resistant bacteria (Doan 1956). Fears have been expressed that dairy foods made from milk containing antibiotics may sometimes contain antibiotic resistant strains of infectious bacteria like Staphylococcus aureus which can cause food poisoning (Albright et al 1961; WHO 1968).

The references cited in this section (3.2) do not convincingly indicate that the antibiotic residues in milk and other foods contribute as significantly to the development of antibiotic resistant microbial populations as other sources.

3.3 Toxic effects: These are mainly associated with high therapeutic dosages and the small amounts of antibiotic residues commonly found in food are unlikely to cause toxic effects (Mol 1975) with the possible exception of chloramphenicol (WHO 1968).

3.4 Other aspects of public health importance: It has been postulated that antibiotic residues can mask pathogenic bacteria possibly by retarding their growth so that the pathogens are not easily detected within the normal time limit of examination procedures (Van Keulen 1965; Mol 1975). It is conceived that this may be of some significance where pathogens, such as salmonella, are present in antibiotic contaminated milk but the possibility of such an event is not high.

Small quantities of antibiotics, as in the case of penicillin residues in milk, administered for a long time can cause considerable

anatomical changes in the intestinal wall which may unfavourably influence the defence system against pathogenic micro-organisms, while the reduction of the normal microflora by the antibiotic residues can lead to overgrowth by resistant pathogens resulting in superinfections (Van Keulen 1965; Mol 1975; Doan 1956).

4. Effect of Heat on Antibiotic Residues in Milk

The effect of pasteurisation on antibiotic residues in milk has been studied by some workers. Katznelson and Hood (1948); Hunter 1949a and Krienke and Fouts (1950) demonstrated that pasteurisation of milk at 145°F for 30 minutes has no effect on penicillin activity. Hunter 1949b showed that temperatures of 150-160°F did not appreciably reduce the activity of penicillin. Generally, the pasteurisation times and temperatures commonly employed in milk processing are inadequate for the destruction or inactivation of penicillin (Doan 1950; Claubauch and Nelson 1951; Coleman and Siegel 1955; Marth and Ellickson 1959).

Pasteurisation does not destroy streptomycin (Hargrove et al 1950). Similarly Edwards and Haskins (1953) observed that milk containing known concentrations of streptomycin, auromycin and penicillin did not show any antibiotic potency loss on heating at 62°C for 30 minutes. Overby (1952) demonstrated that aureomycin, chloromycetin, streptomycin and terramycin in milk were not inactivated when the milk was momentarily heated to 80°C in a waterbath.

Penicillin has been found to be heat stable even at higher temperatures. Watts and McLeod (1946) showed that solutions of penicillin in milk heated to 100°C did not result in any destruction

of the antibiotic within 15 minutes but after 30 and 60 minutes antibiotic loss of 50% and 75% respectively occurred. Coleman and Siegel (1955) showed that boiling of penicillin for 16 hours did not completely destroy its allergenic properties nor did boiling for 5 minutes cause any significant reduction in its activity. Marth and Ellickson (1959), in their review, indicated that a portion of the penicillin activity still remained after either boiling for 60 minutes or autoclaving at 15 lb/square inch steam pressure for 15 to 30 minutes.

Loss of antibiotic activity following pasteurisation of milk has been observed by some workers. Shahani, Gould, Weiser and Slatter (1954) obtained 10-17% antibiotic activity loss when milk containing penicillin, streptomycin and aureomycin was pasteurised at 143°F for 30 minutes. The three antibiotics were inactivated more or less to the same extent at that temperature but streptomycin was more heat labile than penicillin and aureomycin at higher temperatures.

Complete inactivation of penicillin in milk occurred at 250°F, 200°F, 190°F and 160°F for 25, 230, 420 and 1705 minutes respectively (Shahani, Gould, Weiser, and Slatter 1956). At 160°F streptomycin, aureomycin and terramycin in milk were completely inactivated in 1320, 280 and 190 minutes respectively, while at 175°F and 185°F terramycin was inactivated in 92 and 60 minutes respectively and when autoclaved at 15 lb/square inch steam pressure it was completely inactivated within 5 minutes (Shahani, Gould, Weiser, and Slatter 1958).

Heat inactivation of antibiotics in milk is influenced by several factors. It is directly related to the temperatures applied and the length of holding time (Shahani et al 1954; Shahani, Gould, Weiser and Slatter 1955; Shahani et al 1956)

but there is no direct relationship between the concentration of the antibiotic and the degree of inactivation (Shahani et al 1956; Shahani et al 1958). Total solids content of milk appear to have a protective effect (Shahani et al 1958). It was observed that, upon heating at 160°F for 30 minutes, 26% of terramycin was lost in milk with a 10% total solids compared to a 16% antibiotic loss in milk containing 25% total solids. The PH of the milk may also have some influence (Bohnos, Dornbush, Feldman, Martin, Pelcak and Williams 1953/54). They showed that, during heating, terramycin, chlortetracycline and oxytetracycline were less labile at a lower PH than under neutral or alkaline conditions. Heating at 100°C for 15 minutes in solutions at PH 7 or 9 resulted in a greater destruction of oxytetracycline than the other two.

It has been shown that even closely related antibiotics show different heat stabilities (Shahani et al 1954; Shahani et al 1956). These workers observed that different types of either streptomycin or penicillin in milk varied greatly in their heat stability. This is a possible indication that besides other physical and chemical properties, the molecular configuration could also be a determining factor.

Generally, antibiotics are more heat stable in milk than in water or buffer solution. This was reported for chlortetracycline (Shahani et al 1954), aureomycin (Shahani et al 1955) and terramycin (Shahani et al 1958).

MATERIALS AND METHODS

1. Excretion of oxytetracycline and penicillin residues in bovine milk, the correlation between concentrations in the serum and the duration of detectability of residues in milk, following single intramuscular injections.

1.1 Experimental Animals

Three milking cows, with no previous history of antibiotic therapy, were obtained from the Royal (Dick) School of Veterinary Studies, Easter Bush Farm, at the Veterinary Field Station. Milking was carried out at 7.00 am and 3.30 pm each day. Milk production, stage of lactation and identification of each cow were noted and recorded as shown in the table below.

Identification (cow number and breed)	Milk Production (kg/day) - at the beginning of experiment	Stage of lactation (days after last calving)	Drug injected and dosage
B 20 Ayrshire	23.0	81 109	Oxytetracycline: 1200 mg/cow Penicillin: 5 mega units (3 gm)/cow
A 18 Friesian X Ayrshire	17.5	78	Oxytetracycline: (1200 mg/cow)
E 28 Ayrshire	15.1	291	Penicillin: 5 mega units (3 gm)/cow

1.2 Drugs and dosages used

Commercial preparations of oxytetracycline hydrochloride (Engem ycin) and penicillin (Crystapen) were used. Using the dosages shown in the table above, the antibiotics were injected intramuscularly. Two cows were used for each of the two antibiotics but however, as shown in the table, cow No B 20 was used twice with an interval of approximately 4 weeks between oxytetracycline and penicillin injections. The drugs were administered soon after the 7.00 am milking.

1.3 Collection of milk samples

Each experimental cow was milked completely into a separate bucket and the milk thoroughly mixed before sampling. Taking all necessary precaution to avoid contamination sampling was carried out using clean conical flasks and dippers. Control samples were taken immediately before the cows were injected with the antibiotics whereas the test samples were collected during milking times following the injections. All the samples were assayed within 18 hours from the time of sampling. Sampling and testing were continued until negative (absence of residues) results were obtained from at least two consecutive milkings.

1.4 Collection of blood samples and separation of serum

Like milk samples, blood control samples were collected immediately before the administration of the drugs whereas the test samples were collected during milking times. Using vacutainers tubes 20 mls of blood were collected from the tail vein during each sampling. The samples were kept at room temperature ($20^{\circ}C$) for approximately one hour and then kept refrigerated at $4^{\circ}C$ for 18-24 hours. Serum was separated by centrifuging at the rate of 2000 revolutions per minute for 15 minutes, pipetted into a sterile universal bottle and immediately

assayed for antibiotics using the tube dilution method as described below(1:5:2.) Sampling and testing were continued until the antibiotic residues in milk were no longer detectable.

1.5 Methods of Testing

1:5:1 Milk: Two methods were used. (a) Modified disc assay method - cow No B 20 only. The procedure is based on the method of Galesloot and Hassings (1962).

Medium - "ISO-SENSITEST AGAR" (Oxoid, C M 471) prepared in petri dishes.

Preparation of culture An 18-24 hour culture of the Oxford (Heatley) strain of Staphylococcus aureus (NCTC 6571) was used. This was prepared by inoculating 10 mls of nutrient broth with a single colony of the organism picked from a previously inoculated blood agar plate and incubated at 37°C for 18-24 hours.

Inoculating the medium with the culture and standardisation of growth on the plates

The aim was to produce, in all cases, a uniform, nearly confluent lawn of growth covering the whole surface of the medium. Ten-fold dilutions of an 18-24 hour culture, prepared as described above, were prepared using $\frac{1}{4}$ Ringers Solution as diluent. For each dilution volumes ranging between 0.1 and 1.0 ml (for example, 0.1, 0.2, 0.3 .... 1.0) were inoculated and spread evenly over the entire surface of the medium using a sterile applicator. The plates, after drying , were incubated at 37°C for 18-24 hours.

It was found that 0.5 ml of 10^{-3} diluted culture gave the best result.

During the testing periods an 18-24 hour culture, which was available each day, was therefore diluted to 10^{-3} and 0.5 ml of the diluted culture used per each plate.

Testing milk for the antibiotic residues

The milk samples were heated to boiling and cooled immediately under running tap water before testing. Using fine-pointed forceps the edges of a filter paper disc (Antibiotic Assay Filter Paper Discs - Whatman A.A. Discs, 6mm size) were gripped and the disc dipped into the milk sample. As soon as the disc was saturated, the excess milk was removed by pressing the disc against the side of the sample bottle. The disc was placed on the surface of the agar medium ("ISO-SENSITEST AGAR") seeded with a culture of Staphylococcus aureus (Oxford Strain) as outlined above. Three discs per each sample were used. All the plates were incubated at 37°C for 18-24 hours.

Using the control samples various known antibiotic concentrations (standard concentrations) were prepared and treated in the same way as the test samples.

After 18-24 hours incubation period the diameter of the zones of inhibition, where present, was measured and results recorded (Tables 1:5:1(a) and 1:5:1(b)). The diameters of the zones of inhibition of the control samples were plotted against the logarithms of the concentrations. The resultant graph (standard graph) was approximately a straight line (page 39).

The concentration of the antibiotics in the test samples is calculated or obtained from the measurement of the diameter of the zones of inhibition by reference to the standard graph. However, in this experiment, test samples obtained from the first cow (B 20) did not show any zones of inhibition, thus necessatating the use of Bromocresol Purple Test (described below) for the other two cows.

(b) Bromocresol Purple Test (BCP) or "Intertest" (Jacobs, Klasens and Pennings 1972). The procedure for ONE test vial was used. Each test vial contains a lyophilised culture of Streptococcus thermophilus to which known amounts of bromocresol purple indicator, growth promoting and stabilising compounds have been added. When milk containing inhibitory levels of antibiotics is added no growth occurs, the colour of the mixture remains blue and the milk does not clot. In the absence of antibiotics or other inhibitors the growth of the organism occurs producing a range of colour changes from blue through green to yellow and finally coagulation of milk proteins takes place.

Aseptically, a 10 ml portion of well mixed milk sample was poured into a test tube. The tube was closed with a stopper and heated in a boiling water bath until the temperature of the milk reached 95°C. This was indicated by a thermometer in a control tube containing a similar quantity of milk drawn from the same sample. On reaching 95°C the tube was removed from the water bath and cooled in running tap water. The sub-sample was kept refrigerated until tested. Morning samples were tested the same day while samples taken in the afternoon were kept refrigerated after heat treatment until the following day.

The heat treated sub-sample was mixed by inverting several times. The metal closure and rubber seal were removed from a one test vial and using a sterile automatic pipette 2.5 mls of the milk sub-sample were poured into each vial. The rubber seal was replaced and the vial was allowed to stand for 2-3 minutes before inverting gently to mix the contents. The contents were finally transferred into a sterile tube and incubated for 4 hours in water bath operated at 37.5°C. At the end of the incubation period the tubes were removed from the

water bath, inverted once and the colour read by comparing with the colour chart (plate 1) normally supplied with the vials. The control milk samples (samples collected before antibiotic therapy) were tested to rule out the presence of antibiotics and other inhibitors in milk before the drugs were administered. All the results were recorded as shown in Tables 1:5:1(b), 1:5:1(c), and 1:5:1(d).

1:5:2 Serum:Antibiotic concentration in serum was determined by Tube Dilution method as recommended by Garrod, Lambert and O'Grady (1973); Cruickshank, Duguid, Marmion and Swain (1975).

Using the serum samples serial two-fold dilutions in Sterile serum-peptone-water broth, in volumes of 1 ml, were prepared. One-ml blow out pipettes were used - one pipette per dilution. Adequate mixing was ensured before transfer to the next dilution was made.

A known concentration of each of the two antibiotics in the serum control samples was prepared by adding a known quantity of the original antibiotic to a known volume of serum and making further dilutions until the required concentrations were obtained:-

Oxytetracycline - 80 mg/ml of serum

Penicillin - 20 iu/ml of serum

Using these concentrations control series of two-fold dilutions were prepared in the same way as for the test samples. Dilutions ranged between 1/1 and 1/32768 but a control tube containing the medium (serum-peptone-water broth) alone was also included. Both sets of tubes (control and test) were inoculated with a drop (approximately 0.025 ml) of a suspension of an 18-24 hour culture of Staphylococcus aureus (Oxford Strain) diluted to 10^{-3} in nutrient broth. All the samples were incubated at $37^{\circ}C$ for 18-24 hours. After this period the highest dilution showing no turbidity (growth

inhibition) was recorded and the antibiotic concentration in the original serum sample calculated using the formula of Cruickshank et al (1975).

"$\frac{x}{y} \times c$" where the test sample inhibits growth at a dilution of 1 in "x" and the control sample containing "c" µg antibiotic per ml does so at a dilution of 1 in "y".

Results were tabulated as shown in Tables 1:5:1(c) 1:5:1(d) together with results showing excretion and duration of detectability of the residues in milk.

2. Effect of Heat (boiling) on Oxytetracycline and Penicillin Residues in Milk

Milk was obtained from healthy cows known to have had no antibiotic or any other drug therapy for at least 3 weeks before the time of sampling. A one litre conical flask was used. The milk sample was heated to boiling on an electric cooker and then cooled under running tap water.

To ensure that the milk sample was free from any detectable antibiotic or other inhibitory residues a 10 ml sub-sample was obtained and tested for antibiotic residues and other inhibitory substances using Bromocresol Purple Test (BCP) (Jacobs et al 1972).

2.1 Oxytetracycline: Using the milk sample various antibiotic concentrations in milk were prepared in volumes of 50 mls in conical flasks as detailed in Table 1. 25 mls from each of the 50 ml-solutions were aseptically transferred into sterile conical flasks and refrigerated until tested. These sub-samples were labelled Not Heat Treated (NHT). The remaining 25 mls (in conical flasks) were heated to boiling (approximately 100°C) on an electric cooker and maintained boiling for 10 minutes. After the end of this period heating was discontinued and the milk sub-samples cooled immediately under running tap water. These were labelled Heat Treated (HT).

Antibiotic assay was carried out on both sets of sub-samples (NHT and HT) using Modified Disc Assay Method (Galesloot et al 1962) as outlined in section 1:5:1(a). Two discs in separate agar plates were used for each sub-sample in each set. After the incubation period of 18-24 hours at 37°C the diameter of the zones of inhibition was measured (Table 2:1).

2.2 Penicillin: Crystapen in powder form was reconstituted with 18 mls of injectable water (pyrogen free) to make a concentration of 250,000 iu/ml. Taking this as the original solution various antibiotic concentrations using the milk sample were prepared in volumes of 50 mls as follows:-

2 mls Original solution (250,000 iu/ml)	+	18 mls distilled water	→ Solution A (25,000 iu/ml)
1 ml Solution A	+	249 mls milk sample	→ Solution B (100 iu/ml)
25 mls Solution B	+	225 mls milk sample	→ Solution C (10 iu/ml)

				Concentrations
(Control)		50 mls milk sample	→	0.0 iu/ml
10 mls Solution C	+	40 " " "		2.0 "
20 " "	+	30 " " "		4.0 "
30 " "	+	20 " " "		6.0 "
40 " "	+	10 " " "		8.0 "
50 " "	-	-		10.0 "
6 mls Solution B	+	44 " " "		12.0 "
7 " "	+	43 " " "		14.0 "
8 " "	+	42 " " "		16.0 "
9 " "	+	41 " " "		18.0 "
10 " "	+	40 " " "		20.0 "
12.5 "	+	37.5 " "		25.0 "
15.0 "	+	35 " " "		30.0 "

After preparing the antibiotic milk solutions of the required concentrations the same procedure and the same assay method as outlined in 2:1 were followed and the results recorded as shown in Table 2:2; photographs showing zones of inhibition before and after heat treatment of some of the samples are shown (plate 2).

Table 1

Preparation of dilutions - oxytetracycline

2 mls of oxytetracycline (original) (50,000 µg/ml + 998 mls of the milk sample → Solution W (Working solution) 100 µg/ml

Volume of Solution W (working solution) (mls)	Volume of the milk sample (mls)	Total volume (mls)	Concentrations (µg/ml)
50 mls	-	50	100
45	5 mls	50	90
40	10	50	80
35	15	50	70
30	20	50	60
25	25	50	50
20	30	50	40
15	35	50	30
10	40	50	20
5	45	50	10
-	50	50	0

RESULTS

Table 1:5:1(a)

Cow No 1 - Diameter of zones of inhibition of the standard concentrations (Oxytetracycline)

Concentrations Mg/ml	Log (Concentrations)	Mean diameter of zones of inhibition (mm)
0.5	-	0.0
1.0	-	0.0
1.5	0.176	1.0
2.0	0.301	1.5
2.5	0.398	1.8
3.0	0.477	2.0
3.5	0.544	2.3
4.0	0.602	2.5
4.5	0.653	2.7
5.0	0.699	2.8

The diameters of the zones of inhibition Vs logarithms of the antibiotic concentrations ⟶ approximately a straight line graph

(see next page, figure 1)


Diameter of zones of inhibition (mm)
0
1
2
3
.1
.2
.3
.4
.5
.6
.7
Log (Oxytetracycline concentrations in milk)
µg/ml

FIGURE 1

PLATE 1

Bromocresol Purple Test (Intertest)

Colour chart

PLATE 1

Intervet

INTERTEST COLOUR CHART

WARNING

To avoid fading of colours store colour chart away from light when not in use. Always use the fresh chart provided in each pack.

DATE: 15 JUL 1980

INTERVET LABORATORIES LTD. SCIENCE PARK,
MILTON ROAD, CAMBRIDGE, CB4 4BH. TEL. CAMBRIDGE 311221

Table 1:5:1(b)

Oxytetracycline and Penicillin residues in milk following intramuscular injections:

Cow No B 20

Antibiotic	Sample Identification (date and time of collection)	Method of Assay	
		Disc Assay Method	Bromocresol Purple Test
Oxytetracycline	*7-7-80 am	No growth inhibition	
	7-7-80 pm	ND	
	8-7-80 am	ND	
	8-7-80 pm	ND	
	9-7-80 am	ND	
	9-7-80 pm	ND	
Penicillin (Crystapen)	*4-8-80 am	No growth inhibition	Pass
	4-8-80 pm	ND	Fail
	5-8-80 am	ND	Fail
	5-8-80 pm	ND	Pass
	6-8-80 am	ND	Pass
	6-8-80 pm	ND	Pass

* Control Samples taken before antibiotic therapy

ND - Not detectable

Pass - no detectable antibiotic residues. Colour changes from blue through green to yellow.

Fail - presence of antibiotic residues in detectable concentrations. No colour changes, remains blue.

Table 1:5:1(c)

Oxytetracycline residues in milk and serum levels following intramuscular injection.

Cow No A 18

Sample Identification (date and time of sampling)	Serum - Tube Dilution Test		Milk - Bromocresol Purple Test
	Highest dilution inhibiting growth	Concentration of antibiotic in the sample (mg/ml)	
11-8-80 am*	1/512	80.0	Pass
11-8-80 pm	1/32	$\frac{32 \times 80}{512}$=5.0	Fail
12-8-80 am	1/16	$\frac{16 \times 80}{512}$=2.5	Fail
12-8-80 pm	1/16	$\frac{16 \times 80}{512}$=2.5	Pass
13-8-80 am	1/8	$\frac{8 \times 80}{512}$=1.25	Pass
13-8-80 pm	1/4	$\frac{4 \times 80}{512}$=0.625	Pass

* Control sample collected before antibiotic therapy

Table 1:5:1(d)

Penicillin residues in milk and serum levels following intra-muscular injection.

Cow No E 28

Sample Identification (date and time of sampling)	Serum - Tube Dilution Test		Milk - Bromocresol Purple Test
	Highest dilution inhibiting growth	Concentration of antibiotic in the sample (iu/ml)	
18-8-80 am*	1/2048	20.0	Pass
18-8-80 pm	1/128	1.25	Fail
19-8-80 am	1/16	0.16	Fail
19-8-80 pm	1/8	0.08	Pass
20-8-80 am	1/8	0.08	Pass
20-8-80 pm	1/4	0.04	Pass

* Control sample taken before antibiotic therapy

Table 2:1

Effect of heat on Oxytetracycline in milk

Concentration of oxytetracycline in milk (µg/ml)	Diameter of zones of inhibition (mm)						Loss of antibiotic activity (%)
	HT Samples			NHT Samples			
	Plate 1	Plate 2	Mean	Plate 1	Plate 2	Mean	
100	6.5	6.0	6.25	9.0	9.0	9.0	30.6
90	6.0	-	6.0	9.0	8.0	8.5	29.4
80	6.0	5.0	5.5	8.0	8.5	8.25	33.3
70	5.5	5.0	5.25	8.0	8.0	8.0	34.4
60	5.0	5.0	5.0	7.5	8.0	7.75	35.5
50	4.5	4.5	4.5	7.0	7.5	7.25	37.9
40	4.5	3.5	4.0	6.0	7.0	6.5	38.5
30	3.0	3.5	3.25	6.0	6.0	6.0	45.8
20	3.0	3.0	3.0	6.0	5.0	5.5	45.6
10	2.0	3.0	2.5	4.5	5.0	4.75	47.4

HT - Heat treated; NHT - Not heat treated

Loss of antibiotic activity was calculated as follows:

$$\frac{\text{NHT mean} - \text{HT mean}}{\text{NHT mean}} \times 100$$

Calculated mean antibiotic activity loss = 37.84%

Table 2:2

Effect of heat on Penicillin in Milk

Concentration of Penicillin in milk (iu/ml)	Diameter of zones of inhibition (mm)						Loss of antibiotic activity (%)
	HT Samples			NHT Samples			
	Plate 1	Plate 2	Mean	Plate 1	Plate 2	Mean	
2	7.5	7.0	7.25	7.0	7.5	7.25	0.0
4	10.5	10.5	10.5	11.0	10.0	10.5	0.0
6	11.5	11.5	11.5	11.0	12.0	11.5	0.0
8	12.0	12.0	12.0	11.5	13.0	12.25	2.1 (-)
10	13.5	13.5	13.5	13.5	13.0	13.25	1.9
12	14.0	13.5	13.75	14.0	13.5	13.75	0.0
14	14.0	14.0	14.0	14.0	14.0	14.0	0.0
16	14.5	14.0	14.25	14.5	14.5	14.5	1.8 (-)
18	15.0	14.5	14.75	14.0	15.0	14.5	1.7
20	15.0	15.0	15.0	15.0	15.5	15.25	1.7 (-)
25	15.0	15.5	15.25	15.5	15.5	15.5	1.6 (-)
30	16.0	16.0	16.0	15.5	16.0	15.75	1.6

HT - Heat treated; NHT - Not heat treated

(-) - HT Samples with larger zones of inhibition than NHT samples

PLATE 2 (A-E)

The effect of heat (boiling at 100°C for 10 minutes) on known concentrations of oxytetracycline and penicillin added to milk:

Top left - penicillin, heat treated

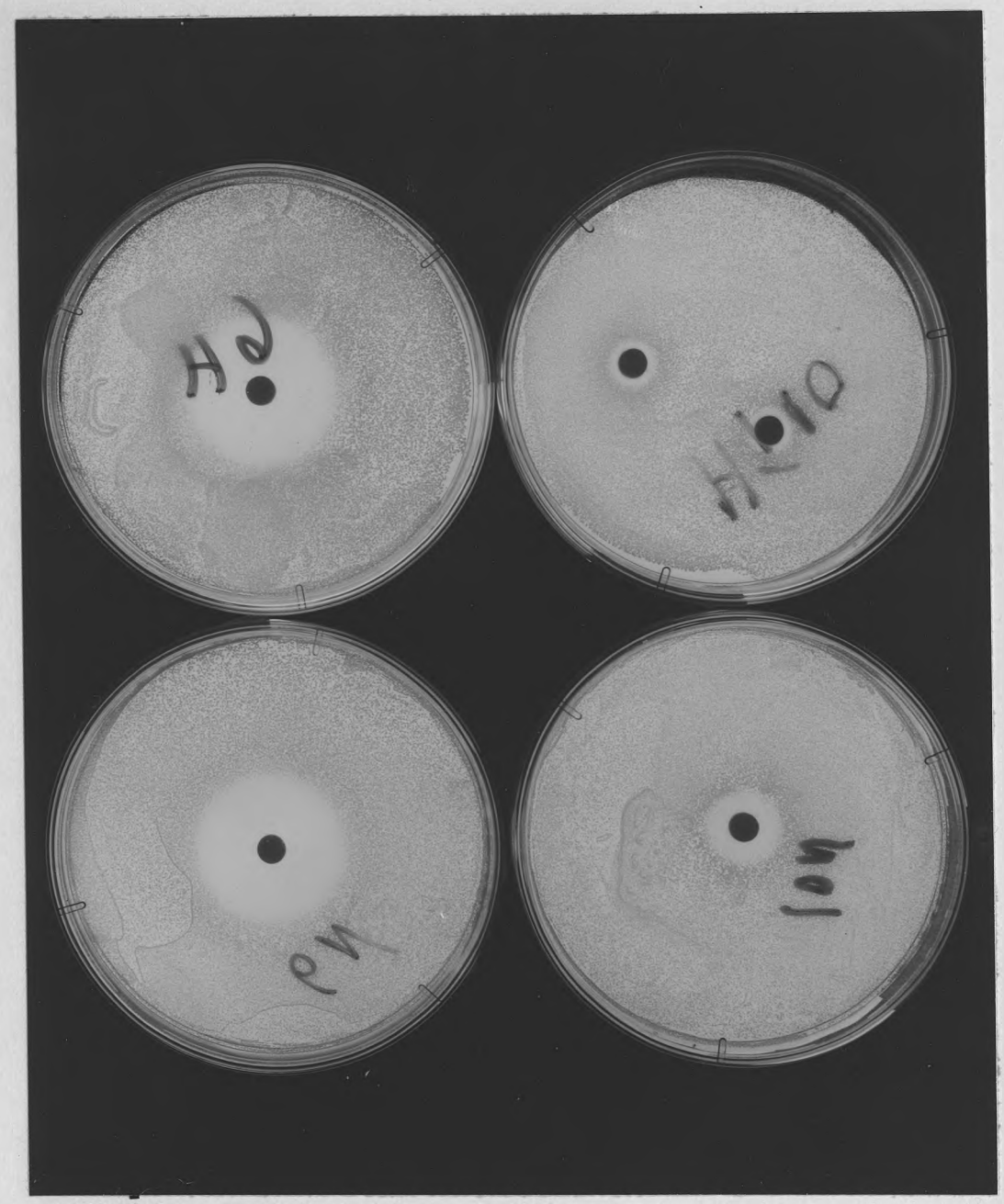
Top right - oxytetracycline, heat treated

Bottom left - penicillin, not heat treated

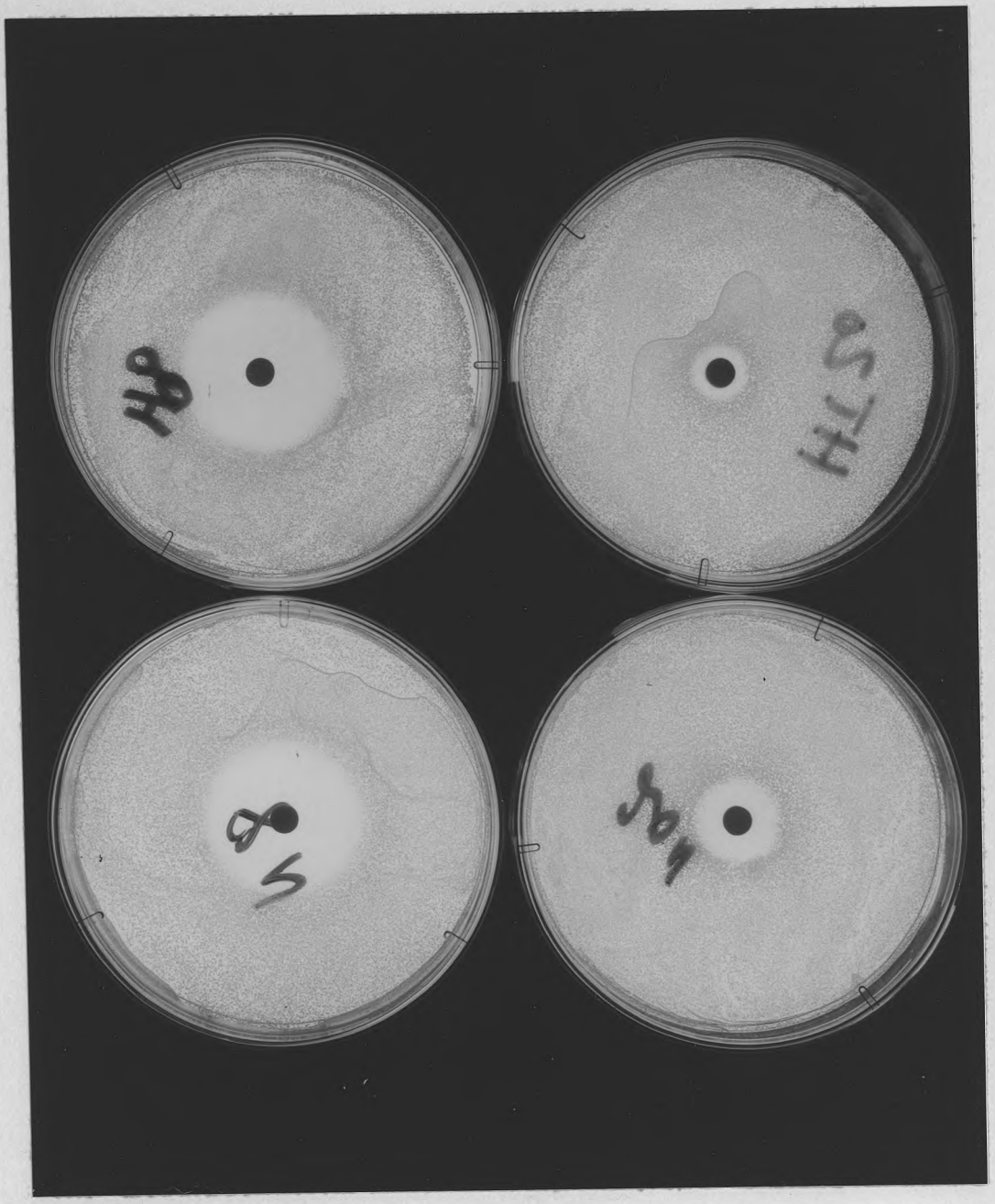
Bottom right - oxytetracycline, not heat treated

	A	B	C	D	E	
Oxytetracycline	10	20	50	60	70	µg/ml
Penicillin	6	8	14	16	25	iu/ml

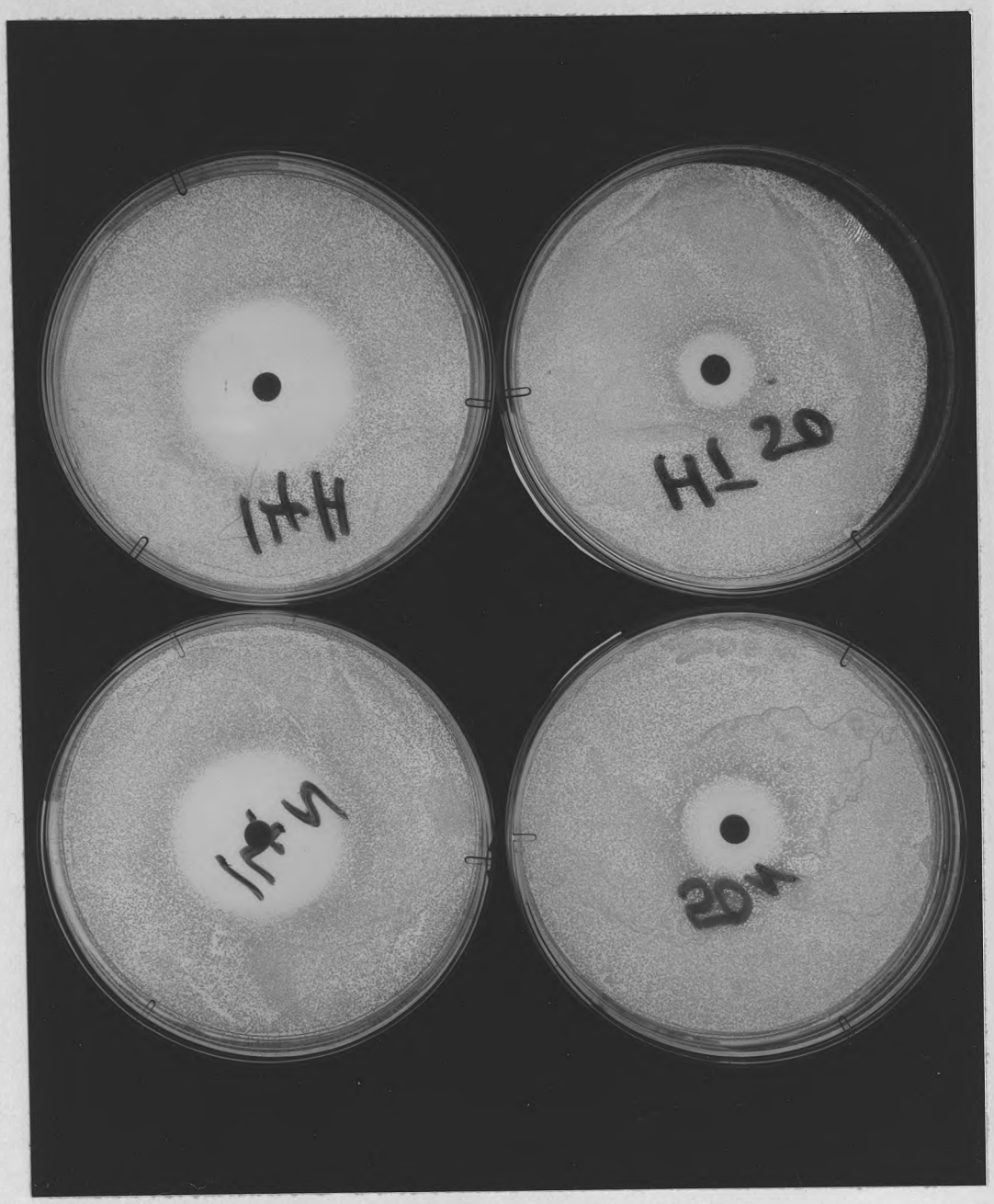
PLATE 2



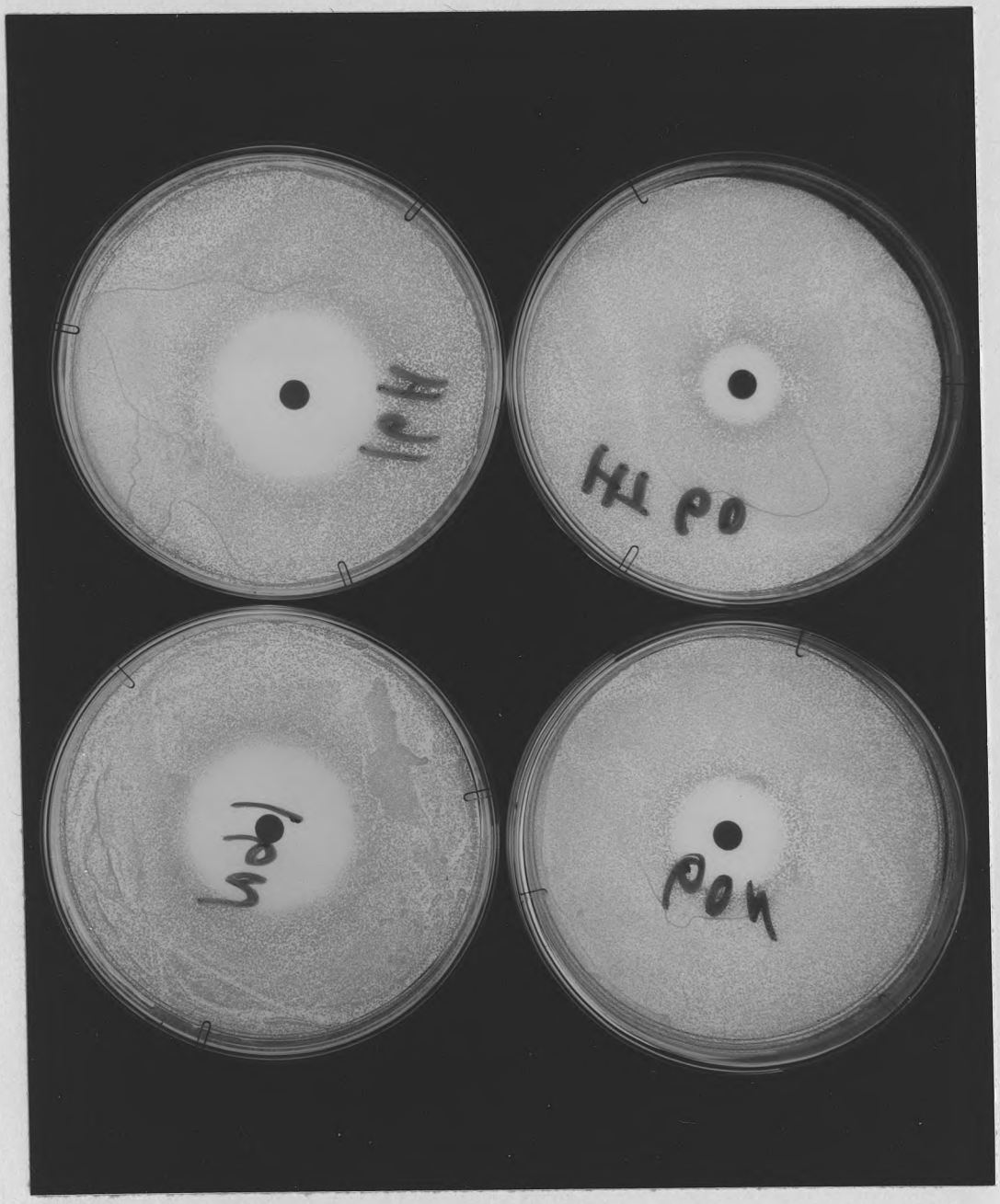
A



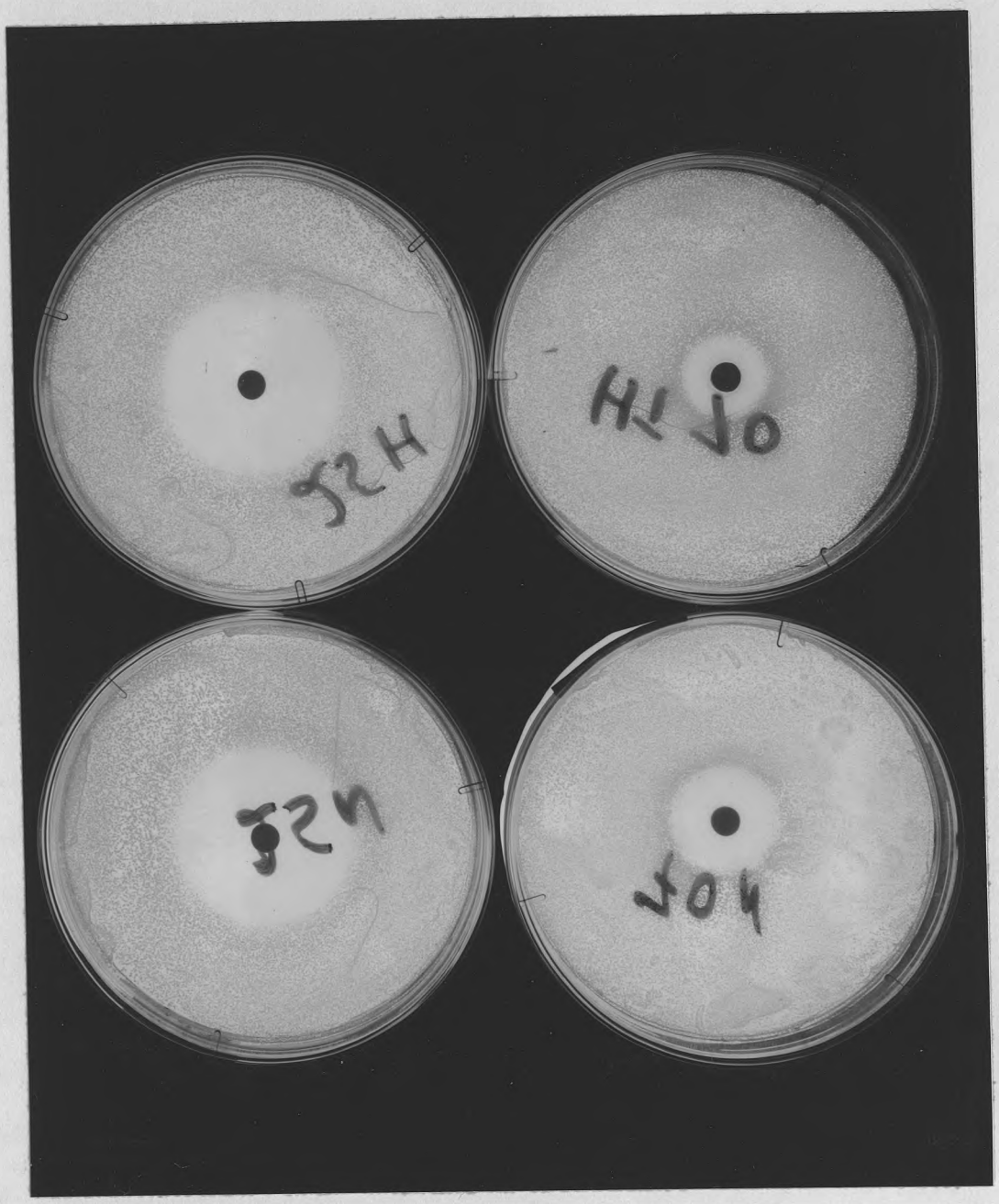
B



C



D



E

DISCUSSION

1. Excretion of oxytetracycline and penicillin residues in milk following intramuscular injections

1:1 Oxytetracycline: The results presented in table 1:5:1(b) indicate that, following an intramuscular injection of 1200 mg of oxytetracycline hydrochloride per cow, residues were not detected in milk using the described disc assay method. The sensitivity of the test was 1.5 µg of oxytetracycline hydrochloride per ml of milk (Table 1:5:1(a). This result agrees with the findings of Barnes (1956) who administered oxytetracycline intramuscularly at the dosage of 1 gm/cow resulting in no detectable residues in the milk. The sensitivity of the test was 1.25 µg/ml. However, this is not an indication that residues were not excreted in the milk. Mol (1975), using a more sensitive test, detected oxytetracycline residues in milk at peak concentrations of 0.4 and 0.9 µg/ml following intramuscular administration of 1.25 and 2.5 gm/cow respectively.

1:2 Penicillin: Similarly, with the disc assay method, penicillin (crystapen) residues were not detected in milk following an intramuscular injection of 5 mega units (3 gm) per cow but nevertheless residues were detected in the milk using a bromocresol purple test, an indication that the milk contained residues but not in concentrations high enough to be detected by the described disc assay method.

It is considered that both antibiotics were excreted in milk following their administration but that the disc assay method employed was not sensitive enough to detect the residues in the milk. The test organism, the Oxford (Heatley) strain of Staphylococcus aureus (NCTC 6571),

used in this method, is reported to have a sensitivity of 0.063 unit of penicillin/ml of milk (Myers 1964) and 0.03 unit of penicillin/ml of body fluid (Cruickshank et al 1975), and therefore less sensitive than Streptococcus thermophilus - the test organism employed in the bromocresol purple test. The latter test was used for cows nos A18 and E28 (tables 1:5:1(c) and 1:5:1(d)).

With the bromocresol purple test both penicillin and oxytetracycline were detected in the milk following their intramuscular administration. The sensitivity of the test is 0.01 iu and 0.5 mg of penicillin and tetracycline per ml of milk respectively (Jacobs et al 1972). It can therefore be deduced that the concentration of oxytetracycline residues in the milk of cow A18 was between 0.5 and 1.5 mg/ml. Similarly, the concentration of penicillin residues in the milk of cow E28 was not less than 0.01 units per ml. Both drugs were therefore shown by the bromocresol purple test to be excreted in the milk following single intramuscular administration at the recommended dosages.

1:3 Duration of detectability of the residues in milk (Tables 1:5:1(c) and 1:5:1(d)) - both oxytetracycline and penicillin

The milk samples collected 32 hours (3rd milking) after injection were free from detectable residues but samples collected during the second milking (24 hours) following administration of the antibiotics were positive for the presence of the residues. The maximum duration of detectability of the residues for both drugs was therefore between 24 and 32 hours. This agrees in part with the findings of Williams and Laverne (1960) who obtained a clearance time of 30 hours following intramuscular injection of tetracycline

hydrochloride at the dosage of 2 mg/lb body wt. However, their observation that procaine penicillin at the dosage of 2,000 units/lb body wt. resulted in a clearance time of 2 days does not wholly agree with the observation made in this experiment. Mol (1975) obtained a clearance time of 2 days following a single intramuscular injection of 1.25 g of an aqueous solution of oxytetracycline per cow while Sadek (1954), using pronapen at the dosage of 5000 units/lb body wt. obtained a mean clearance time of 24 hours. Edwards (1966) obtained a penicillin level of 0.06 iu/ml of milk 24 hours following an intramuscular injection of 5 mega units in aqueous form.

Different durations of detectability of residues and their peak concentrations in milk have been reported by different workers. These differences are due to a variety of factors which include different types of preparations of the same drug, different dosages, tests of different sensitivities, animals of different milk production, variations between individual animals, etc, some of which are not easy to standardise. However, in many cases, the differences observed are of very little practical importance and need not be of major concern.

1:4 The correlation between concentrations in the serum and the duration of detectability of residues in milk

1:4:1 Oxytetracycline (Table 1:5:1(c)): The data presented in Table 1:5:1(c) indicate that the last milk sample to show the presence of residues was collected 24 hours (2nd milking) after the antibiotic administration. The serum sample collected at the same time had an oxytetracycline concentration of 2.5 µg/ml. However, the milk sample

collected during the 3rd milking following injection was free from detectable residues despite the fact that the antibiotic concentration in the corresponding serum sample was still 2.5 mg/ml. This is an indication that the blood antibiotic concentration of 2.5 mg/ml hardly gave rise to the excretion of residues in milk and that some of the residues detected in the 2nd milking sample following injection must have been excreted in the milk when the concentration in blood was higher than 2.5 mg/ml. It can therefore be stated that serum levels of more than 2.5 mg of oxytetracycline per ml result in the excretion of residues in the milk. Schipper and Petersen (1953) obtained a maximum blood concentration of 0.3 mg/ml following an intramuscular injection of 3 gm of terramycin per cow but no residues were detected in the milk.

1:4:2 Penicillin (Table 1:5:1(d)): The last milk sample in which residues were detected was collected 24 hours (2nd milking) following injection. The serum sample collected at the same time had penicillin concentration of approximately 0.16 iu/ml. This milk started accumulating in the udder since the previous milking when penicillin concentration in the blood was 1.25 iu/ml and therefore some of the residues must have been excreted in milk when the blood level was in the range 0.16-1.25 iu of penicillin/ml. It is evident that a blood concentration of 0.08 iu of penicillin per ml did not result in detectable residues in the milk since the milk samples of 19/8/80 pm and 20/8/80 am did not show residues while the serum level remained at 0.08 iu of penicillin/ml. However, serum levels in the range 0.08-0.16 iu of penicillin/ml would possibly result in detectable residues in the milk. It appears therefore that the serum level of 0.16 iu of penicillin per ml was the critical level that resulted in excretion of residues in

the milk. This compares favourably with the results obtained by Edwards (1966). He found that, after intramuscular injection of 1 mega unit of procaine penicillin in aqueous and oil forms, the penicillin levels in the milk 7 hours following injections were 0.04 and 0.02 iu/ml while the concentrations in the blood were 0.35 and 0.25 iu/ml respectively.

2. Effect of heat (boiling) on oxytetracycline and penicillin in milk - tables 2:1; 2:2

The data recorded in table 2:1 show that oxytetracycline was partially inactivated following boiling of the milk at 100°C for 10 minutes. A mean antibiotic activity loss of 37.84% was obtained with a tendency for the antibiotic activity loss to be higher in lower than in higher antibiotic concentrations. This suggests that the antibiotic would be more heat stable in the original solution than when added to milk. Other workers have also observed a partial antibiotic activity loss of tetracyclines (chlortetracycline, terramycin) following heat treatment at various time/temperature combinations (Shahani et al 1954; Shahani et al 1955. Shahani et al 1958). These workers have further observed that the rate of antibiotic inactivation is directly related to the amount of heat applied and that there is no relationship between the concentration of the antibiotic and the degree of inactivation.

Penicillin on the other hand (table 2.2) was not inactivated to any extent following a similar heat treatment as for oxytetracycline. This confirms the findings of Watts and McLeod (1946) who showed that penicillin in milk heated to 100°C did not result in any destruction of the antibiotic within 15 minutes.

In this experiment, penicillin was shown to be more heat stable than oxytetracycline. At very high temperatures both antibiotics could possible be inactivated completely but such temperature/time combinations may have no practical application in the field of milk technology.

Boiling of milk is quite common amongst many rural communities, especially in the developing countries, and this explains the relatively low incidence of milk borne infections in those areas (Skovgaard 1965). However, the partial inactivation of antibiotic residues likely to occur when milk is boiled or heat treated in some other ways is not considered to be of great value in overcoming the problem of antibiotic residues in milk. This is particularly so with penicillin, which unfortunately, is the most important antibiotic of public health significance because of its allergenicity even in low concentrations.

CONCLUSIONS

Excretion of oxytetracycline and penicillin in milk following single intramuscular injections was demonstrated. Antibiotic contamination of milk resulting from this source (administration by injection) may be more than is actually thought. Whereas many farmers appreciate that drugs are excreted in milk following intramammary infusions very few can appreciate that drug residues are excreted in milk following administration by injections. In the latter case instructions regarding withholding periods are not taken seriously and therefore more violations occur. This is of particular significance in areas where antibiotics are used indiscriminately.

The duration of detectability of antibiotic residues in milk depends on many factors some of which are pertinent to the drug and others to the individual animal concerned. After injection the drug diffuses from the inoculation site into the blood and then into the milk via blood/milk barrier. Although the antibiotic concentration in blood is an important parameter in determining the concentration in milk other factors like protein binding, nature of the drug, mode of administration, health status of the animal, etc, are of importance in governing the excretion process. Therefore, a simple correlation between antibiotic levels in blood and in milk cannot be stated. To be of any practical value the determination of the duration of detectability of residues should be carried out on many animals selected at random. Since this has the advantage of correcting for variable factors the calculated mean value would be more meaningful in determining duration of withdrawal periods than any result obtained from a single trial.

From the public health point of view antibiotic residues in milk and other foods are important for two main reasons: (a) allergic reactions in a few individuals who are sensitised to penicillins, which are the antibiotics mainly responsible for allergic manifestations, and (b) development of antibiotic resistant pathogens when micro-organisms are frequently exposed to such residues. Whilst cases of allergic reactions resulting from the consumption of milk containing penicillin residues have been reported by several workers, the extent to which antibiotic residues in milk influence the occurrence of antibiotic resistant pathogens is not easy to define.

Regardless of public health considerations antibiotic residues in milk are undesirable for other reasons. As a result of their interference with the growth of starter cultures used in the manufacture of fermented products the dairy industry can experience heavy financial loss.

As far as the heat treatment of milk is concerned, it can be concluded that neither boiling at 100°C for 10 minutes nor current method(s) of pasteurisation are adequate for the total destruction of antibiotic residues. Further, the time/temperature combinations, such as those indicated in the literature, which would be required for complete inactivation of residues may not be of any practical application in many situations.

There is no simple solution to the problem of antibiotic residues in milk. Each of the parties involved, namely the drug manufacturer, the user and the milk producer, has a part to play and it is necessary that each of them understands his responsibilities in this matter. Of prime importance is the co-operation of the milk

producer. It is essential to remind him of the necessity to observe the instructions given regarding the withdrawal period. This could be achieved by initiating Milk Producer Courses which, besides other aspects of milk production, could incorporate relevant topics on antibiotic contamination of milk. This, in addition to testing schemes with price penalties for milk found to contain antibiotics, could reduce the incidence of antibiotic contamination of milk significantly.

Surveys of milk to determine the extent of antibiotic residues need to be encouraged. This is fundamental to the solution of the problem. It is considered that unless the problem is well defined success in formulating and implementing preventive measures would be limited.

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